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16. ABSTRACT

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Comparative Study of Various Methods Used for Determining Health Effects of Inhaled Sulfates

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Various health effect parameters were compared to determine which tests were the most sensitive indicators of toxic effects of exposure to metallic sulfate aerosols. Inhalation studies were conducted involving either single 3-hr exposure to various concentrations of cupric sulfate (0.43–2.64 mg/m³ SO_4), aluminum sulfate (1.65–2.75 mg/m³ SO_4), and aluminum ammonium sulfate (1.47–3.81 mg/m³ SO_4) or multiple (five and ten) daily 3-hr exposures to cupric sulfate (0.1 mg/m³ SO_4). The test parameters studied in male and female CD_1 mice were changes in mortality after respiratory infection with Group C *Streptococcus zooepidemicus;* pulmonary bactericidal activity; pulmonary cell number, type, viability, and ATP content; and pulmonary morphology by scanning electron microscopy. Tracheal ciliary beating frequency and morphology were also studied in both CD_1 mice and Syrian golden hamsters. Differences in bacteria-induced mortality rate appeared to be the most sensitive and consistent indicators of pollutant damage. The other parameters produced evidence of damage but generally only at the higher pollutant concentrations. Cupric sulfate was the most toxic of the three sulfates, but the differences between the toxicity of the aluminum sulfate and aluminum ammonium sulfate were less clear. © 1986 Academic Press, Inc.

INTRODUCTION

Sulfates constitute a substantial fraction of particles in polluted air. Experimental evidence suggests that particulate sulfates may contribute significantly to health hazards (Rall, 1974). Thus it is important to determine functional, morphological, and host defense consequences of inhaled sulfates on the respiratory system. The studies described here were designed to assess a spectrum of *in vivo/in vitro* assays to provide toxicity data on three particulate air pollutants: cupric sulfate ($CuSO_4$), aluminum sulfate $Al_2(SO_4)_3$, and aluminum ammonium sulfate ($Al_2(SO_4)_3(NH_4)_2SO_4$). These chemicals were selected since previous work in our laboratory (Ehrlich, 1980) had indicated that these compounds would produce varying degrees of toxicity. The health effects were evaluated after a single 3-hr exposure to each of the three sulfates or multiple (five and ten) daily exposures to $CuSO_4$. The parameters evaluated were changes in resistance to respiratory infection; number, type, viability, and adenosine triphosphate (ATP) content of pulmonary free cells; pulmonary bactericidal activity; tracheal ciliary

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beating frequency and morphology; and morphology of the pulmonary system as observed by scanning electron microscopy. Data from each of the parameters were correlated to determine which were the most sensitive indicators of the toxic effects of the sulfates.

MATERIALS AND METHODS

Animals. The 3- to 4-week-old male and female CD_1 mice (Charles River Breeding Laboratories, Portage, Mich.) and the 4-week-old male Syrian golden hamsters (Ela:ENG(Syr), ARS/Sprague–Dawley, Madison, Wisc.) used in these studies were quarantined for 7 to 14 days prior to the experiments. During the pollutant exposures and infectious challenge, the animals were held in individual compartments of stainless steel wire-mesh cages. At other times, the animals were group housed in plastic shoe box cages. Feed (Rodent Laboratory Chow 5001, Ralston Purina Co., St. Louis, Mo.) and water were available *ad libitum*, except during pollutant exposure and infectious challenge.

Inhalation exposures. Air pollutant exposures were carried out in 432-liter Plexiglas chambers supplied with filtered room air. Air temperature and relative humidity monitored during the exposures ranged from 23 to 30°C and from 30 to 50% RH. Deotized cage boards placed on the floor of the chambers prevented the build-up of ammonia during the exposure.

The pollutant aerosols were generated as described by Ehrlich (1980) with a Model 40 DeVilbiss nebulizer containing 1% (w/v) aqueous solutions of $CuSO_4$ (J. T. Baker Chemical Co., Phillipsburg, N.J.), $Al_2(SO_4)_3 \cdot 18H_2O$ (Fisher Scientific Co., Fair Lawn, N.J.) and $Al_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$, (Fisher Scientific Co.). The sulfate solutions were replenished at 30-min intervals during the 3-hr exposure. The aerosol was passed through a heated glass tube for drying.

The aerosol mass concentration in the chamber was determined from two replicate samples collected over the 3-hr exposure period on 47-mm diameter 0.2-μm Millipore filters. Collection flow rates were controlled by 2.0-lpm critical orifices which had been previously calibrated with a wet-test meter. The collected sulfate samples were assayed as $BaSO_4$ by a turbidimetric method (Public Health Services, 1965) and mg/m^3 of sulfate (SO_4) was determined. Metal concentrations were calculated from these values.

Particle size was determined with an eight-stage cascade impactor (Inhalation Toxicology Research Institute, Albuquerque, N.M.) only for the 2.16-mg/m^3 $Al_2(SO_4)_3(NH_4)_2SO_4$ aerosol. The amount of SO_4 collected on each impactor stage was assayed by the $BaSO_4$ turbidimetric method and was used to determine the mass median aerodynamic diameter (MMAD) and geometric standard deviation (g) of the aerosol. For this exposure, the MMAD was 0.75 μm with a g of 2.00. Although actual measurements were not made during the other acute exposures, the aerosol generation and delivery systems were identical in all experiments so that the particle size was expected to be similar during all exposures. During one of the $CuSO_4$ aerosol exposures, particle size determined with a Quartz crystal microbalance (California Measurements, Inc., Serra Madre, Calif.) gave a MMAD of 0.54 μm with a g of 2.07.

Animals were exposed to the experimental atmospheres or clean filtered air for a single 3-hr period or, in the multiple exposures, for 3 hr/day, 5 days/week for 1 or 2 weeks. Because of limited chamber size, replicate exposures for the various parameters were carried out on 2 or more days. Health effect assays were conducted within 1 hr after the final exposure.

Host defense assays. For the infectivity model (Ehrlich *et al.*, 1977), mice exposed to the pollutant and control mice exposed to filtered air were simultaneously challenged with an aerosol of *Streptococcus zooepidemicus*. The inhaled dose of streptococcus was determined by colony count in lungs homogenates of four or five mice immediately after the infectious challenge. The inhaled dose averaged 10 colony forming units per mouse. After the infectious challenge, the mice were housed in a clean-air, isolated animal room for a 14-day observation period, during which deaths were recorded daily. The mean survival time (MST) was calculated as described by Pindak *et al.* (1971).

Pulmonary free cells were obtained by tracheobronchial lavage and total and differential cell counts, viability, and cellular ATP levels were determined as previously described (Aranyi *et al.*, 1983).

Bactericidal activity of alveolar macrophages was determined in the lungs of individual mice using aerosols of ^{35}S-labeled *Klebsiella pneumoniae* disseminated with a Retec X-70 disposable nebulizer (Aranyi *et al.*, 1983). Three mice from the 4-hr postbacterial exposure group were sacrificed, lungs homogenized, and the number of viable bacteria then was related to the number of viable plus nonviable bacteria as determined by scintillation counting.

Tracheal explants. The methods for obtaining and evaluating effects tracheal explants were described by Schiff *et al.*, (1979a, b) and are briefly summarized here. The tracheas were rinsed in Hanks' balanced salt solution and cut transversely into rings approximately 1 mm. The tracheal rings were placed in plastic petri dishes containing 0.7 ml of CMRL 1066 medium supplemented with 0.2% bovine albumin, penicillin (250 U/ml), and streptomycin (250 μg/ml).

Effects of the exposures were expressed as alterations in ciliary activity and cytopathology. Cilia beating frequency at the periphery of each ring explant was determined with an electronic stroboscope as the light source. Cilia beating frequency was measured at each quadrant of the lumen and the measurements were averaged and recorded as beats per minute (bpm). The percentage of normal epithelium, defined as a smooth luminal surface with beating cilia, was determined for each tracheal ring.

For histopathology studies, tracheal ring explants from the animals were immediately fixed in neutral buffered Formalin, dehydrated, cleared, and embedded in paraffin, and sections (5 μm) were cut and stained with hematoxylin and eosin. Tracheal specimens for scanning electron microscopic examination were fixed with Karnovsky's paraformaldehyde–glutaraladhyde phosphate-buffered fixative, critical-point dried, and coated with gold (Ketels *et al.*, 1977).

Statistical analysis. Mortality data were analyzed using log-linear models (Bishop *et al.*, 1975; Haberman, 1979). These statistical models which are appropriate for qualitative responses, such as mortality, were used in this study to

examine the effects of concentration, exposure, and sex on mortality incidence. In addition to the additive effects of these design variables we also tested the following interactions:

(1) concentration by exposure;
(2) concentration by exposure by sex.

In the multiple-exposure experiments, a term for number of exposures was substituted for the pollutant concentration term, and a corresponding log-linear model was fitted to the mortality data.

Student's t test was used to determine the statistical significance of treatment differences in mean survival time, pulmonary bactericidal activity, and pulmonary free cell parameters. These test statistics were corrected for the problem of multiple comparisons using the Bonferoni:inequality correction. Hypotheses involving cilia beating frequency were tested by two-way analysis of variance with Duncan's New Multiple-Range Test being used to detect treatment differences. Treatment differences in percentage normal epithelium were determined from the χ^2 distribution. Because no between-sex differences in response were detected in two tracheal epithelial measurements (cilia beat frequency and percent normal epithelium) in the single exposure studies, data from both sexes were combined for statistical analysis in those studies, but not for the multiple-exposure studies.

RESULTS

Air pollutant concentrations for the single 3-hr exposures were selected with the aim of providing a range of responses for each pollutant. For the three test compounds, the mean and range of the sulfate concentrations during the 3-hr exposures are shown in Table 1. For discussion purposes, the levels will be referred to as "high," "intermediate," or "low."

Mortality. A single 3-hr exposure to an aerosol of $CuSO_4$ at any of the test concentrations significantly increased the mortality rate and decreased the MST of both male and female mice challenged with streptococcus (Table 2). The magnitude of the response appeared to be directly related to the pollutant concentration.

Additional analysis of the single-exposure $CuSO_4$ data by log-linear modeling revealed a highly significant ($P < 0.001$) concentration–response relationship.

TABLE 1
MEAN AND RANGE OF SULFATE CONCENTRATIONS DURING THE SINGLE 3-hr EXPOSURES TO THE TEST COMPOUNDS

Compound	Sulfate concentration (mg/m³)					
	High		Intermediate		Low	
	Mean	Range	Mean	Range	Mean	Range
$CuSO_4$	2.53	2.41–264	0.93	0.75–1.13	0.43	—
$Al_2(SO_4)_3$	2.71	2.66–2.75	2.31	2.26–2.36	1.84	1.65–2.02
$Al_2(SO_4)_3(NH_4)_2SO_4$	3.79	3.76–3.81	2.10	1.95–2.25	1.47	—

Differences in mortality between the control and pollutant-exposed mice increased linearly with increasing pollutant concentration. There was a significant ($P < 0.05$) between-sex difference in overall mortality, but not in the concentration–response relationship; that is, the increasing difference in mortality between control and pollutant-exposed mice with increasing metal sulfate concentration was the same for both sexes.

Exposure to the high concentration $Al_2(SO_4)_3$ significantly increased the mortality rate and decreased the survival time only for female mice challenged with streptococcus. Data from the intermediate and low concentrations of $Al_2(SO_4)_3$ were quite similar and the between-group differences were not significant. Results of the log-linear analysis of the $Al_2(SO_4)_3$ mortality data paralleled those with that of $CuSO_4$. There was a significant ($P < 0.05$) concentration–response relationship and overall between-sex difference ($P < 0.05$) in mortality, but again there was no sex difference in the concentration–response.

The mortality rate was increased and MST was decreased in all groups of mice exposed to $Al_2(SO_4)_3(NH_4)_2SO_4$ and then challenged with the infectious aerosol. However, the differences between treatment groups were, in general, not significant. When the data were subjected to log-linear analysis, the only significant effect on mortality was a main effect of experimental group; i.e., the aerosol-exposed mice had a higher mortality rate ($P < 0.05$) when averaged over both sexes

TABLE 2

CHANGES IN MORTALITY AND MEAN SURVIVAL TIME OF CD_1 MICE AFTER A SINGLE 3-hr EXPOSURE TO SULFATE SALTS AND CHALLENGE WITH AEROSOLS OF STREPTOCOCCUS

Pollutant	mg SO_4/m³ (calculated mg metal/m³)	Sex	Number of mice		Change in mortality[a] (%)	Change in mean survival time[a] (days)
			Control	SO_4		
$CuSO_4$	2.53	M	24	23	+100**	−11.0**
	(3.30)	F	24	24	+100**	−11.0**
	0.93	M	98	100	+61.7**	−4.9**
	(1.21)	F	97	100	+77.6**	−6.7**
	0.43	M	24	24	+54.2**	−4.2**
	(0.56)	F	24	23	+70.3**	−5.9**
$Al_2(SO_4)_3$	2.71	M	57	56	+18.1	−1.5*
	(0.51)	F	57	54	+39.6**	−3.5**
	2.31	M	47	61	+4.8	−0.9
	(0.43)	F	48	62	+18.6	−1.4
	1.84	M	24	24	+4.1	−1.0
	(0.34)	F	24	24	+20.8	−1.8
$Al_2(SO_4)_3(NH_4)_2SO_4$	3.79	M	24	24	+16.7	−1.3
	(0.53)	F	24	23	+13.2	−1.2
	2.10	M	48	49	+5.2	−0.8
	(0.30)	F	48	50	+27.0*	−2.3**
	1.47	M	24	24	+16.7	−1.5
	(0.21)	F	24	24	+8.3	−1.0

[a] Sulfate-exposed minus control animals. Significant difference between sulfate-exposed and corresponding infected control groups. (Bonferoni adjusted probability values, *$P < 0.05$, **$P < 0.01$).

TABLE 3
CHANGES IN MORTALITY AND MEAN SURVIVAL TIME OF CD_1 MICE EXPOSED TO CUPRIC SULFATE 3 hr/DAY, 5 DAYS/WEEK FOR 1 OR 2 WEEKS AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

Number of exposures	mg SO_4/m^3 (calculated mgCu/m^3)		Sex	Number of mice		Mortality (%)	Change in mean survival time[a] (days)
	Mean	SD		Con	SO_4		
5	0.09	0.01	M	48	47	−3.9	+0.5
	(0.12)		F	48	48	−2.1	+0.1
10	0.10	0.02	M	48	48	+31.2**	−1.5*
	(0.13)		F	48	48	+25.0**	−1.3*

[a] Sulfate-exposed minus control animals. Significant difference between sulfate-exposed and corresponding infected control groups (*$P < 0.05$, **$P < 0.01$).

and the three concentrations. The concentration–response was not linear and there were no between-sex differences.

In the multiple-exposure studies (Table 3), no changes in mortality were observed when mice were challenged with streptococcus aerosol following five daily 3-hr exposures to $CuSO_4$ (approximately 0.12 mg Cu/m^3). However, both male and female mice challenged with the infectious agent after 10 daily exposures had a significantly increased mortality rate and decreased MST. Similarly, with the log-linear analysis, mortality was significantly increased ($P < 0.05$) with increased exposure to $CuSO_4$ but there were no between-sex differences in mortality.

Pulmonary free cells. Effects of single 3-hr exposures to aerosols of various metal sulfates on pulmonary free cells, differential counts and viability of the pulmonary free cells (not shown in tables) were not found in any of the exposure conditions. Generally, greater than 96% of the lavaged cells were viable and approximately 98% were alveolar macrophages. However, as shown in Table 4, within 1 hr of a single exposure to the high concentration of $CuSO_4$ the only significant change observe ' in the pulmonary free cell population was a decreased total cell count in the male mice. However, 24 hr after the exposure, total cell counts were significantly reduced, and cellular ATP levels were elevated in both sexes. At the intermediate concentration of $CuSO_4$, no significant differences were detected either within 1 hr of or 24 hr after exposure. Multiple exposures to aerosols of $CuSO_4$ (Table 5) did not result in significant changes in pulmonary free cell numbers or ATP levels.

After exposure to two concentrations of $Al_2(SO_4)_3$, total cell counts and cellular ATP levels were not affected (Table 4). At the low $Al_2(SO_4)_3$ concentration, pulmonary free cells parameters were only measured within 1 hr following exposure. No between-group differences were observed.

No significant changes in total cell counts were detected in mice exposed to $Al_2(SO_4)_3(NH_4)_2SO_4$ (Table 4). However, both male and female mice did show significantly increased pulmonary cellular ATP levels immediately after exposure to the high concentration of $Al_2(SO_4)_3(NH_4)_2SO_4$.

TABLE 4

EFFECTS OF SINGLE 3-hr EXPOSURE TO AEROSOLS OF SULFATE SALTS ON PULMONARY BACTERICIDAL ACTIVITY

Pollutant	mg SO_4/m^3 (Calculated mg metal/m^3)	Interval between exposure and assay (hr)	Sex	Pulmonary free cells: Total cells (count × 10^5) n^a	Total cells (count × 10^5) %[b]	Cellular ATP (10^8 fg/10^5 cells) n^a	Cellular ATP (10^8 fg/10^5 cells) %[b]	Bactericidal activity: *K. pneumoniae* killed n^a	Bactericidal activity: *K. pneumoniae* killed %[b]
$CuSO_4$	2.53 (3.30)	<1	M	26	42.0**	20	74.6	24	61.0**
			F	25	79.0	23	83.5	24	57.1**
		24	M	24	36.5**	23	144.0**		N.D.[c]
			F	24	60.6*	21	181.0*		N.D.
	0.93 (1.21)	<1	M	38	98.0	33	113.5	44	98.8
			F	36	92.4	30	102.4	44	97.5
		24	M	36	88.5	31	105.9		N.D.
			F		N.D.		N.D.		
$Al_2(SO_4)_3$	2.71 (0.51)	<1	M	16	127.7	16	96.6	44	104.8*
			F	18	73.5	16	102.2	47	113.6**
		24	M	34	81.0	30	116.4		N.D.
			F	34	80.7	32	97.8		N.D.
	1.84 (0.34)	<1	M	16	65.4	16	95.2	23	100.5
			F	16	105.8	16	90.2	23	96.6
$Al_2(SO_4)_3(NH_4)_2SO_4$	3.79 (0.53)	<1	M	16	79.3	15	170.1**		N.D.
			F	16	122.6	16	213.6**		N.D.
	2.10 (0.30)	<1	M	16	200.9	16	89.3	20	127.7**
			F	16	111.7	16	121.6	18	113.1
		24	M	32	84.3	31	79.8		N.D.
			F	32	126.8	32	92.0		N.D.

[a] Number of mice.

[b] Percentage of control value. Significantly different than corresponding control mice exposed to filtered air (Bonferoni adjusted probability values, $*P < 0.05$, $**P < 0.01$).

[c] N.D., not done.

Pulmonary bactericidal activity. Pulmonary bactericidal activity was significantly reduced in both male and female mice exposed once for 3 hr to the high concentration of $CuSO_4$, but was not affected by exposure to the intermediate concentration (Table 4). A single exposure to $Al_2(SO_4)_3$ at the high concentration resulted in significantly increased bactericidal activity. However, no changes were observed after exposure to the low concentration. Pulmonary bactericidal activity was also significantly increased in male mice exposed to the intermediate level of $Al_2(SO_4)_3(NH_4)_2SO_4$. The effects of the high concentration were not determined.

Multiple exposures to aerosol of $CuSO_4$ (Table 5) resulted in significantly decreased bactericidal activity in female mice after 5 daily 3-hr exposures, and in both sexes after 10 daily exposures.

Tracheal epithelium. The effects of single 3-hr exposure to the metal sulfate aerosols on cilia beating frequency and percentage normal epithelium are shown in Table 6. Significantly decreased cilia beating frequency was seen only in hamsters exposed to the high concentrations of $CuSO_4$ or $Al_2(SO_4)_3(NH_4)_2SO_4$. Cytologic alterations were observed in the tracheal epithelium of sulfate-exposed hamsters. Significant decreases in percentage normal epithelium were seen in hamsters after exposure to $CuSO_4$, $Al_2(SO_4)_3$, and $Al_2(SO_4)_3(NH_4)_2SO_4$. No exposure-related changes in percentage normal epithelium were observed in mice, suggesting that hamster tracheal epithelium is more sensitive to pollutant exposure. It is important to note, however, that the percentage of normal epithelium of the mouse was smaller than that in the hamster and in one case, cellular debris did not permit observations in the mouse.

Microscopic examination of the mouse trachea after the 3-hr exposures to metal sulfate aerosols revealed epithelia similar to those of the control mice. A common observation in both control and pollutant-exposed mice was the exfoliation of differentiated surface epithelial cells from the mucosa. However, in pol-

TABLE 5

EFFECTS OF MULTIPLE DAILY 3-hr EXPOSURES TO CUPRIC SULFATE AEROSOL ON PULMONARY FREE CELLS AND BACTERICIDAL ACTIVITY

Exposure				Pulmonary free cells				Bactericidal activity *K. pneumoniae* killed	
				Total cells (count × 10^5)		Cellular ATP (10^8 fg/10^5 cells)			
mg SO_4/m³ (Calculated mg metal/m³) Mean	SD	Number of exposures (3 hr/day)	Sex	n^a	%[b]	n^a	%[b]	n^a	%[b]
0.09 (0.12)	0.01	5	M	17	100.6	15	86.5		N.D.[c]
		5	F	17	71.1	16	92.9		N.D.
0.09 (0.12)	0.03	5	M		N.D.		N.D.	22	102.0
		5	F		N.D.		N.D.	24	94.2*
0.10 (0.13)	0.02	10	M	16	75.8	15	91.3	22	94.5*
		10	F	17	82.5	17	101.8	18	85.2**

[a] Number of mice.

[b] Percentage of control. Significantly different than corresponding control mice exposed to filtered air. (*$P < 0.05$, **$P < 0.01$).

[c] N.D., not done.

TABLE 6
CILIA BEATING FREQUENCY AND PERCENTAGE NORMAL EPITHELIUM IN HAMSTER AND MOUSE TRACHEA IMMEDIATELY AFTER A SINGLE 3-hr EXPOSURE TO SULFATES

Pollutant	SO_4 Conc. (mg/m^3)	Mean cilia[a] (beats/min)				Normal epithelium[b] (%)			
		Mouse		Hamster		Mouse		Hamster	
		Air	SO_4	Air	SO_4	Air	SO_4	Air	SO_4
$CuSO_4$	2.53	1370	1359	1248	1153*	62	60	75	51*
	0.93	1306	1293	1216	1225	55	49	62	56
$Al_2(SO_4)_3$	2.71	1396	1388	1222	1209	—[c]	—[c]	80	55*
	1.84	N.D.[d]	N.D.	1256	1203	N.D.	N.D.	91	75*
$Al_2(SO_4)_3(NH_4)_2SO_4$	3.79	N.D.	N.D.	1216	1143*	N.D.	N.D.	93	59*
	2.10	1322	1320	1261	1231	77	65	93	86

[a] Mean of 48 observations of 12 tracheal rings from four animals.
[b] Mean of 12 tracheal rings from four animals. Normal tracheal epithelium is defined as a smooth surface with beating cilia.
[c] Bubbling of epithelium with debris present; cilia movement was visible and appeared normal, but quantitative evaluation could not be made.
[d] N.D., not done.
* Significant difference from control animals exposed to filtered air ($P < 0.05$).

lutant-exposed mice the percentage of desquamation was greater. Cytoplasmic vacuolation was seen in approximately 10% of the epithelium after pollutant exposure. The tracheas from the control hamsters showed a surface epithelium of normal pseudostratified ciliated columnar appearance with an occasional loss of cilia. After exposure to $CuSO_4$ or $Al_2(SO_4)_3$, approximately 75% of the tracheas had an epithelium similar to the controls. Moderate epithelial flattening, focal areas of exfoliation of surface epithelial cells, and focal epithelial ulceration were seen in some specimens.

Scanning electron microscopic examination revealed a tracheal epithelial surface consisting of three distinct cell types: ciliated, microvillous, and goblet or mucous secreting. Tracheas from control hamsters and mice had knob-like structures on the distal end of the microvilli and fully formed ciliated cells. Patchy and isolated areas of unciliated cells were seen in the hamsters, while the tracheal tissue of the control mice was more uniform in appearance. Hamster tracheal epithelium removed and fixed immediately after exposure to the higher level of cupric or aluminum ammonium sulfate had increased amounts of mucous covering the surface of the microvillous cells. The epithelial surfaces of tracheas from mice exposed to sulfates were unchanged.

Table 7 shows the effects of multiple 3-hr daily exposures to $CuSO_4$ on the tracheal epithelium. Following five exposures to the aerosol, male mice showed a significant reduction in percentage normal epithelium compared to the controls. However, because of the relatively small amount (40%) of normal epithelium in the control mice, this change is of questionable biological significance. After 10 exposures to $CuSO_4$ female, but not male, mice showed reduced normal epithelium. In these mice, 25% of the epithelial surface had a jagged appearance due to exfoliating cells.

TABLE 7
CILIA BEATING FREQUENCY AND PERCENTAGE NORMAL EPITHELIUM IN HAMSTER AND MOUSE TRACHEA IMMEDIATELY AFTER FIVE AND TEN 3-hr EXPOSURES TO CUPRIC SULFATE

No. of exposures	SO_4 concentration (mg/m^3)		Mean Cilia[a] (beats/min, hamster[c])	Normal epithelium (%)[b]		
	Mean	SD		Mouse		
				Male	Female	Hamster
5	0	(Filtered air)	1263	44	36	98
	0.09	0.01	1213	30*	28	89
10	0	(Filtered air)	1227	73	83	97
	0.10	0.02	1190	71	73*	85

[a] Mean of 48 observations of 12 tracheal rings from four animals.
[b] Mean of 12 tracheal rings from four animals. Normal epithelium is defined as a smooth surface with beating cilia.
[c] Cilia beat in mouse organ culture could not be evaluated due to bubbling of epithelium.
* Significant difference from control animals exposed to filtered air ($P < 0.05$).

No changes in cilia beat frequency were observed in hamster after 5 or 10 exposures. Cytologic and histologic examinations of the tracheal mucosa from the hamsters revealed no treatment-related alterations. Both control and pollutant-exposed hamsters had normal pseudostratified ciliated columnar epithelium. An occasional mucous cyst was observed in hamsters exposed 10 times to the $CuSO_4$. In mice, bubbling of the epithelium and the presence of cellular debris on the mucosal surface made the cytologic and histologic evaluation impossible. Thus the overall quality of the tracheas of CD_1 mice is so poor that it is difficult to detect changes attributable to a test pollutant.

SEM examination of the respiratory tract. SEM examination of mice exposed for 3 hr to $CuSO_4$ (2.53 mg SO_4/m^3) revealed crystalline particles scattered throughout the respiratory system. In the nasal cavities, particles were seen on both ciliated and nonciliated cells. A heavy patchy mucous layer was noted in the trachea. In those areas of the trachea where no mucous was present, the crystalline particles were found attached to, or near, ciliated cells. Within the lung, particles were observed in the bronchi, terminal bronchioles, and alveoli. No cellular destruction was observed, but this was not unexpected since the tissues were obtained shortly after pollutant exposure. No cellular damage was observed in the respiratory tracts of mice exposed to $Al_2(SO_4)_3$ (2.71 mg SO_4/m^3) or $Al_2(SO_4)_3(NH_4)_2SO_4$ (2.10 mg SO_4/m^3).

In the multiple-exposure studies, all control mice had essentially normal respiratory tract structure (Fig. 1A) although alveolar thickening was seen occasionally at the periphery of the lung. The bronchi of mice exposed 5 or 10 times to the $CuSO_4$ (0.1 mg SO_4/m^3) aerosol were normal. After 5 daily exposures, some alveoli were slightly thickened and irregular in appearance (Fig. 1B), while after 10 exposures extensive areas of alveolar thickening were seen with many of the walls fused into irregular masses (Fig. 1C).

DISCUSSION

Exposure to any of the concentrations of $CuSO_4$ used in this study led to a

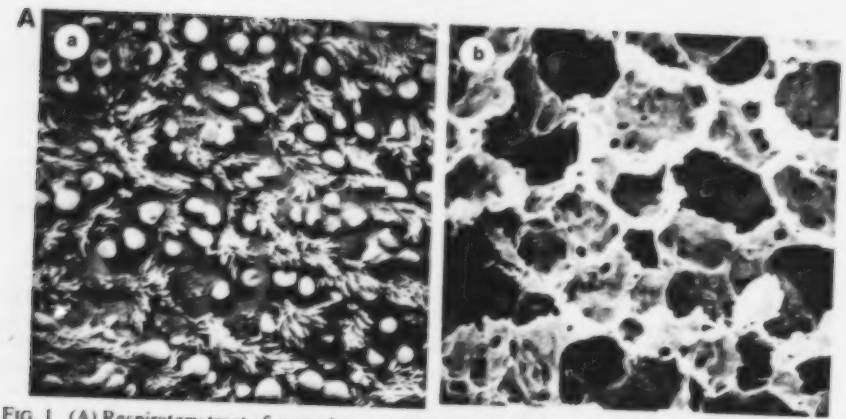

FIG. 1. (A) Respiratory tract of control mouse after five daily 3-hr exposures to filtered room air: (a) bronchus (630×), and (b) alveoli (333×).

significantly increased mortality rate and decreased MST in the CD_1 strain of mice subsequently challenged with streptococcus. These results were similar to those obtained by Ehrlich (1980) in which mice of another strain (CD_2F_1) were exposed to aerosols of $CuSO_4$, also for a single 3-hr period. When compared to their respective filtered-air controls, significant changes were also noted in total pulmonary cell count, ATP levels, and pulmonary bactericidal activity in mice and in percentage normal epithelium in hamsters, but only at the highest concentration studied (2.53 mg SO_4/m^3).

Exposure to $Al_2(SO_4)_3$ also showed an apparent concentration-related effect on the mortality resulting from challenge with the bacterial aerosol. In addition, there was better correlation among the various test parameters compared to the other test pollutants. Again, these mortality data were in reasonable agreement with previous studies by Ehrlich (1980). As was the case with $CuSO_4$, there were between-sex differences in overall mortality, but not in the concentration–response to the pollutant.

In general, as with $CuSO_4$, significant changes in the other quantitative parameters, except cellular ATP levels, were seen at the high concentration of $Al_2(SO_4)_3$ but not at the intermediate concentration. The one exception was reduced percentage normal tracheal epithelial in hamster exposed to the lower concentration.

After exposure to the highest concentration of $Al_2(SO_4)_3(NH_4)_2SO_4$, significant changes between controls and experimental animals were noted in cellular ATP levels in mice and tracheal cilia beating frequency and normal epithelial in hamsters. Although the increased mortality and decreased MST of mice exposed to this highest concentration of $Al_2(SO_4)_3(NH_4)_2SO_4$ were not judged significantly different by the χ^2 test, the log-linear analysis revealed a significant decrease in mortality when averaged over concentration. Unlike the other two pollutants, however, there was no apparent concentration relationship or between-sex difference.

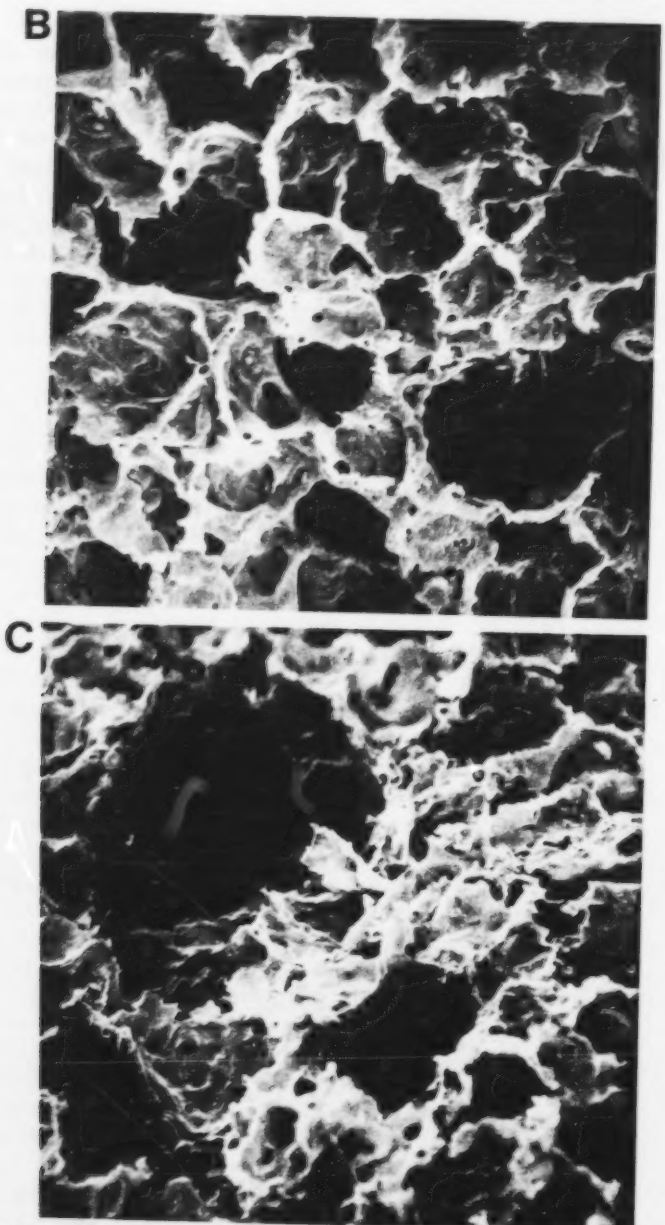

FIG. 1. (B) Alveoli of mouse after 5 daily 3-hr exposures to $CuSO_4$ (0.1 mg/m^3 SO_4). The alveoli were thickened and irregular in appearance. 475×. (C) Alveoli of mouse after 10 daily 3-hr exposures to $CuSO_4$ (0.1 mg/m^3 SO_4). Alveoli were thickened with many of the walls fused into irregular masses. 525×.

The mortality changes elicited between controls and mice exposed to $Al_2(SO_4)_3(NH_4)_2SO_4$ followed by infectious challenge were less than that produced by $Al_2(SO_4)_3$. These results are in agreement with previous work (Ehrlich, 1980) that indicated that $Al_2(SO_4)_3(NH_4)_2SO_4$ was less toxic than $Al_2(SO_4)_3$. However, in the present study no clear dose-response was obtained because of the number of mice used. Interestingly, in a study that compared the effects on susceptibility to respiratory infection of inhaled versus intratracheally injected metals (Hatch *et al.*, 1981), aluminum ammonium sulfate was found to be more effective than aluminum sulfate in increasing susceptibility to infection when both were injected intratracheally, just opposite to the results when both compounds were administered by inhalation. These results suggest that the response to these aluminum compounds is complex and worthy of further investigation.

With the intermediate concentration of $Al_2(SO_4)_3(NH_4)_2SO_4$, effects were seen in mortality rate and MST (females only), and pulmonary bactericidal activity (males only). It should be noted, however, that the exposures to both $Al_2(SO_4)_3$ and $Al_2(SO_4)_3(NH_4)_2SO_4$ resulted in enhanced pulmonary bactericidal activity when compared to filtered-air-exposed controls. In other studies in our laboratory (Aranyi, 1981), enhanced pulmonary bactericidal activity was observed when mice were exposed to pollutant aerosols of relatively low toxicity. These observations parallel the numerous observations concerning the decreased susceptibility to infection from exposure to low concentrations of dusts and probably result from increased pulmonary cell numbers or activity (Casarett, 1971). On the other hand, inhalation of more toxic aerosols appears to overcome this initial activation and depresses the bactericidal ability of the pulmonary macrophages.

Between-pollutant comparison clearly indicated that $CuSO_4$ was the most toxic of the three compounds. Accordingly, it was selected for use in multiple-exposure studies. The exposure concentration for these studies were selected so that the total dose of $CuSO_4$ for the five-exposure study would be, on a concentration $\times$ time (CT) basis, approximately equal to the total dose of the low concentration from the single exposures. After five exposures the only significant effects were increased pulmonary bactericidal activity in female mice and decreased percentage normal epithelium in hamsters. Mortality rate and MST following streptococcal challenge did not differ significantly from the controls even though the total dose (CT) was equivalent to a dose which greatly altered these parameters in the single 3-hr exposure. This indicates that the changes in the host are more related to the concentration of the pollutant. After 10 exposures, increased mortality rate, decreased MST, and decreased pulmonary bactericidal activity were observed in both male and female mice, while decreased percentage normal epithelium was seen only in female mice. However, a significant finding in these studies was the poor quality of the respiratory epithelium in control CD_1 mice. Because of large areas of cellular necrosis with accompanying loss of cilia and desquamation, the tracheas of the CD_1 mice did not appear appropriate for the assessment of air pollutant effects and future studies should be performed in hamsters.

As expected, the qualitative parameters (light and scanning electron microscopy) revealed little evidence of morphologic alterations during the single 3-hr

exposures. However, evidence was obtained of the presence of presumable pollutant particles throughout the respiratory tree. Although no effects were seen in the tracheas of multiple-exposure animals when examined by light or electron microscopy, SEM examination of the respiratory tract revealed an apparently dose-related evidence of damage at the alveolar level.

When the responses of the various health effects parameters are compared across pollutants and number of exposures, the most consistent and sensitive indicators of pollutant damage were the mortality rate and MST of the streptococcal infected mice. This was particularly true in the case of $CuSO_4$, but less clear with the other two pollutants where different health effect parameters appeared to be as sensitive. These variable responses may be indicative of different mechanisms of damage for each pollutant and underscore the efficacy of a battery of different tests for best assessing pollutant damage, particularly when the health effects of the pollutant are unknown. However, the mouse model system probably best represents the overall responses of the respiratory tract to airborne insult and is the single most sensitive indicator of pollutant damage.

Finally, in these studies a number of effects were seen which appeared only in male or in female mice. It can be expected that this susceptibility was not related directly to any potential differences in deposition due to body weight (e.g., lung size) or perhaps to clearance differences. This sex variation highlights the need to evaluate both sexes.

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